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Expression of a Cosmid Containing the LCR, $^{A}\gamma$, δ, and β Globin Genes in Mouse Erythroleukemia Cells

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A novel method involving the packaging of phage particles was used to introduce cosmids containing components of the human β-globin locus control region (LCR) and the human $^{A}\gamma$, δ, and β globin genes into mouse erythroleukemia (MEL) cells. After stable transfection, MEL clones were selected and analyzed for expression of human genes. Both $^{A}\gamma$ and β globin mRNA were expressed in these clones, indicating that MEL cells do not suppress transcription of the human γ globin gene. The variability of human $^{A}\gamma$ vs. β globin expression from clone to clone prevents a clear delineation of differences in the expression of these two genes with a cosmid in which a region potentially involved in γ-to-β switching has been deleted. The results suggest that MEL cells are capable of supporting human γ expression, despite their predominantly adult phenotype. © 1996 Wiley-Liss, Inc.

Key words: cosmid, locus control region, γ globin gene, β globin gene

INTRODUCTION

The locus control region (LCR), located more than 20 kilobases upstream of the human ε globin gene, has been shown to contain important control sequences for globin gene expression [1,2]. Its full activity can be retained by linking four small restriction fragments together to form a micro-LCR (μLCR) [3]. A cosmid construct that contains the μLCR linked to the human $^{A}\gamma$, δ, and β globin genes in their natural context (μLCR$^{A}\gamma\psi\beta\delta\beta$) has been shown to express the human $^{A}\gamma$ and β genes in a developmental stage-specific manner in transgenic mice, i.e., there is primarily $^{A}\gamma$ expression in yolk sac cells, and β expression in mouse fetal liver and adult bone marrow [4]. We sought to determine if this regulation was retained when the cosmid was introduced into erythroid cell lines.

A previous study [5] in which hypersensitive site 2 alone was inserted into the 5′ end of a 40-kilobase cosmid containing the $^{G}\gamma^{A}\gamma^{-117}\delta\beta$ genes in their normal chromosomal organization failed to suppress human γ globin expression in mouse erythroleukemia (MEL) cells, which are presumed capable of expressing primarily adult mouse β globin. In addition, the human globin genes were not significantly inducible. Interpretation of the results in this study was complicated by the fact that the $^{A}\gamma$ gene in the construct contained a known nondeletion HPFH mutation at position −117. Another study examined the expression of transfected gene constructs into human fetal erythroid × mouse erythroleukemia cell hybrids [6]. These hybrids initially expressed human fetal globin genes, but with increasing time in culture expressed adult β globin. Human γ genes transfected into these cells were expressed at all stages of hybrid development. The lack of developmental regulation of these transfected genes was attributed either to the absence of important control sequences such as the LCR in the constructs employed, or to a time-dependent modification of chromosomal DNA, such as methylation.

To further investigate the mechanism of competition between the human γ and β globin genes in the context of these studies, we introduced the μLCR$^{A}\gamma\psi\beta\delta\beta$ construct into MEL cells. This construct contains the wild-type $^{A}\gamma$ gene and representative restriction fragments from the four hypersensitive domains of the LCR. The cosmid was linearized and ligated into pWE15, a vector that contains the neomycin resistance gene as a selectable marker in eukaryotic cells. Efficient transfer of DNA into MEL

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cells was accomplished using in vivo packaging of cosmids into phage particles.

This construct was employed to try to analyze the function of an oligopyrimidine region upstream of the human δ globin gene. A factor present in nuclear extracts from adult mouse and human hematopoietic cells that binds to this region has been identified in our laboratory [7]. The binding site for the factor is within an unusual 250-base pair domain that is >95% pyrimidines on one strand (*pyr* sequence). The position of the site in the β-globin cluster and its potential for adopting an unusual secondary structure suggest that it may play a role in hemoglobin switching. To examine the functional role of this region, a 511-base pair region from −708 to −1219 upstream of the δ globin gene was deleted from the original cosmid construct. The entire cosmid was ligated to pWE15 to allow for stable selection in mouse erythroleukemia cells.

The results of our studies indicate that it is possible to stably introduce cosmids containing and expressing human γ and β globin genes into MEL cells. However, we noted considerable variability in the amount of $^{A}\gamma$ and β globin mRNA expression from clone to clone, using both wild-type and deleted cosmids. This lack of consistent expression of the relative or absolute levels of these genes may be due to either variations in the developmental stage of different MEL clones, or to different chromosomal positions of integration of the cosmid.

MATERIALS AND METHODS

μLCR^{A}γψβδβ-pWE15 Cosmid

The construct μLCR^{A}γψβδβ, which contains a 2.5-kilobase (kb) μLCR cassette linked to the ^{A}γψβδβ globin locus, was a gift of T. Enver (University of Washington). It contains 1,200 base pairs (bp) of the 5′ flanking sequence to the $^{A}\gamma$ gene, and 5,100 bp of the 3′ sequence flanking the β gene. The entire locus had been cloned into a modified version of the pHC79 vector (Fig. 1). The cosmid was linearized with *Not*I restriction enzyme and ligated into pWE15 (Clontech, Palo Alto, CA), a cosmid vector that contains the neomycin resistance gene. The ligation mix was packaged with the Gigapack II Gold packaging extract (Stratagene, La Jolla, CA) and used to transform bacteria. Kanamycin-resistant colonies were grown and analyzed by several different restriction digests. Cosmid DNA was purified by cesium chloride gradient.

μLCR^{A}γψβδβ-del (*pyr* sequence)-pWE15 Cosmid

The construct μLCR^{A}γψβδβ, linked to pHC79 as described above, was modified as follows: A 7.9-kb *Sca*I-*Sal*I restriction fragment was isolated from the cosmid. A 1.4-kb *Age*I-*Sal*I piece was then obtained from this fragment and cloned into a pUC19 vector with a modified polylinker. To perform the necessary deletion, the *Age*I-*Sal*I fragment in the plasmid was cut with *Bsa*BI and *Bsm*I. The ends were filled in with T4 DNA polymerase and religated. The *Age*I-*Sal*I fragment, with 511 base pairs of sequence removed upstream of the δ globin gene, including the *pyr* sequence, was excised from the pUC19 vector. The cosmid was modified by restriction digest with *Age*I, followed by partial digestion with *Sal*I. The 886-base pair *Age*I-*Sal*I fragment was then ligated into the cosmid, and the mixture was packaged and used to transform bacteria. Ampicillin-resistant colonies were grown and analyzed by various restriction digests. Cosmids with the 511 base pair deletion were obtained (Fig. 1). This construct was linearized with *Not*I and ligated into pWE15 vector, as described above.

Packaging of Cosmids In Vivo and Phage Preparation

The cosmid constructs were packaged in vitro as described above, and transduced into DK22 (obtained from ATCC, Rockville, MD), an *Escherichia coli* host that allows thermoinducible repackaging into phage particles [8]. For the preparation of phage particles for gene transfer, DK22 transductants were inoculated into 500 ml of LB medium containing kanamycin (30 μg/ml) and grown at 30°C with shaking until the OD_{600} equaled 0.3. In vivo packaging of phage particles was induced by rapid shift of temperature of the bacteria to 42°C for 20 min, followed by further incubation at 37°C for 3 hr. Release, purification, and titering of phage particles was performed as previously described [9].

Stable Transfection

MEL cells in log phase were collected and washed in 20 volumes of ice-cold phosphate-buffered saline (PBS). The cells were resuspended into aliquots of 10^7 cells, pelleted, and resuspended in 1 ml of calcium phosphate precipitate in 1 × HEPES-buffered saline containing 5 μg cosmid DNA and 15 μg salmon sperm DNA as carrier. The suspension was allowed to sit at room temperature for 20 min. Ten ml of DME medium containing 10% fetal bovine serum and antibiotics were added, and the cells were transferred to tissue culture dishes. The cells were incubated for 18 hr at 37°C in a humidified incubator, and then placed in 24-well plates (Nunc, Naperville, IL). After 48 hr, selection was performed with G418 (Gibco BRL, Gaithersburg, MD) at 800 μg/ml (dry weight). Clones were isolated after 14 days and expanded. For induction, cells were incubated in media containing 1.5% dimethylsulfoxide (DMSO) for 5–7 days.

Alternatively, MEL cells were transfected using bacteriophage particles containing the cosmids. Phage containing the wild-type μLCR^{A}γψβδβ-pWE15 cosmid, or the mutant μLCR^{A}γψβδβ-del (*pyr* seq)-pWE15 cosmid, were transfected into MEL cells by the standard calcium

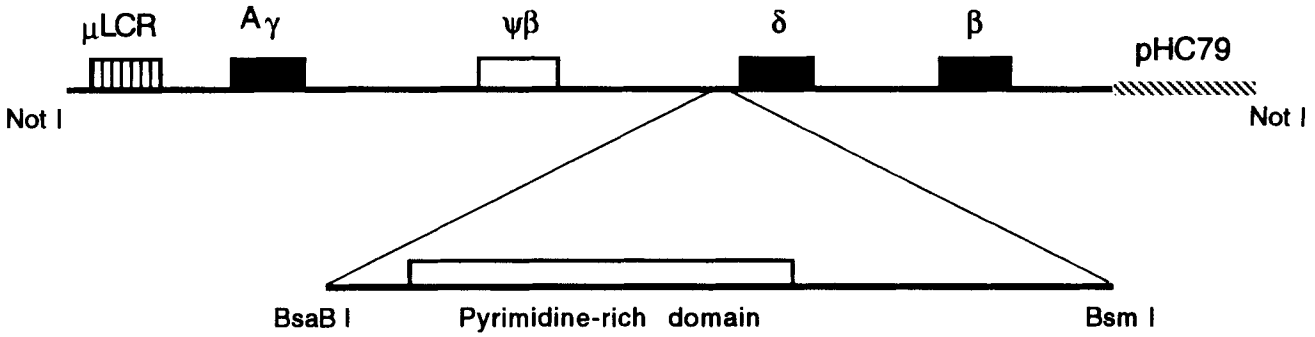

Fig. 1. Construct μLCR^{A}γψβδβ in pHC79 cosmid was linearized at the *Not*I site and ligated into *Not*I-digested pWE15 vector. A 511-base pair *Bsa*BI-*Bsm*I fragment was excised in the μLCR^{A}γψβδβ-del(*pyr* sequence)-pWE15 construct in order to delete the pyrimidine-rich domain upstream of the human δ-globin gene.

phosphate-mediated procedure outlined above, using 5×10^9 phage particles for 10^7 MEL cells. Phage particles containing cosmid were also introduced into MEL cells, using cationic liposomes as a vehicle for particle transfer. MEL cells (2×10^7) were resuspended in 3 ml of Optimem I medium (Gibco BRL), containing 50 μg Lipofectin reagent (Gibco BRL) and 5×10^9 phage particles. The cells were incubated in tissue culture dishes for 6 hr, and then an equal volume of DME containing 20% fetal calf serum and antibiotic/antimycotic was added. After overnight incubation, the cell suspension was diluted with DME medium and transferred to 24-well plates prior to selection with G418.

Isolation of Cytoplasmic RNA

Total cytoplasmic RNA was isolated from individual clones using NP-40 lysis [10] in the presence of placental RNase inhibitor (Strategene). The concentration of RNA was determined by ultraviolet spectroscopy, and the integrity of the RNA by agarose gel electrophoresis. RNA was stored in TE buffer, pH 7.6, at −70°C.

Isolation of Genomic DNA and Southern Blot Analysis

DNA was isolated from MEL cell clones, using standard technique [10]. Ten μg of DNA were digested with *Xba*I and hybridized with a digoxigenin-labelled probe (Boehringer Manheim, Indianapolis, In), using the 0.7-kb *Eco*RI-*Pst*I fragment from the 3′ end of the β globin gene [11]. Signal detection was performed using the manufacturer's instructions.

RNA Analysis by Primer Extension

Primer extension was performed as previously described [12]. Primers for human γ and mouse α were those previously employed [13]. Five μg of total cytoplasmic RNA were employed for MEL and transfected lines. One ng of RNA from human reticulocyte and 20 μg of total cytoplasmic RNA from hemin-induced K562 cells were employed as controls.

RESULTS

Cosmids containing the μLCR and "wild-type" ^{A}γ, δ, and β globin genes, or the construct with the *pyr* sequence deleted as part of a 511-base pair restriction fragment upstream of the δ globin gene, were employed (Fig. 1). These cosmids were then linearized with *Not*I restriction enzyme and ligated to pWE15, a cosmid vector that contains the neomycin resistance gene as a selectable marker in eukaryotic cells. These constructs were transfected into MEL cells, a line that produces adult mouse globin. Stable transfectants were selected with G418, and resistant clones were expanded. The cells were then exposed to 1.5% DMSO for 7 days to induce both mouse and human globin gene expression. The relative abundance of mRNA transcripts for human β and γ globin, as well as for mouse α and β major globins, was determined by the method of primer extension.

The efficiency of gene transfer by calcium phosphate precipitation of the cosmid constructs into MEL cells was very low (approximately 10^{-7} per μg cosmid DNA). In an attempt to obtain higher efficiencies of gene transfer of the cosmid, the use of phage particle-mediated transfer was employed [9,14]. Lambda phage particles were obtained by in vitro packaging of the pWE15-human globin locus ligation mix. These phage particles were transduced into the *E. coli* strain DK22, a thermoinducible in vivo packaging host [8]. The cosmid DNA was then repackaged into phage particles and released from the host bacteria by chloroform lysis. Phage particles were purified by ultracentrifugation in cesium chloride, dialyzed against phage buffer, and sterilized by chloroform treatment [10]. Intact phage particles containing the cosmid DNA were then introduced into eukaryotic cells by standard coprecipitation methods. This method was further optimized

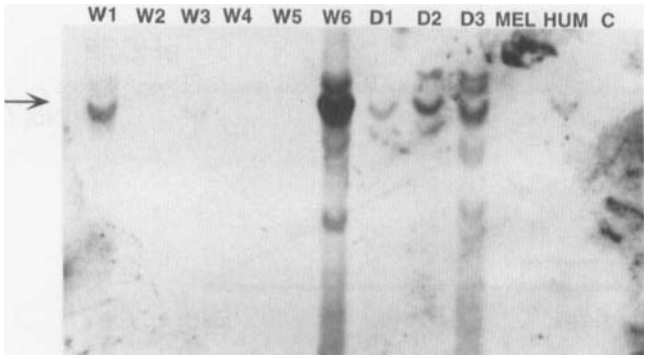

Fig. 2. Southern blot analysis of transfected clones using calcium phosphate precipitation of cosmid DNA. Ten micrograms of *Xba*I-digested genomic DNA were run on a 0.7% agarose gel, blotted onto nylon membrane, and probed with a 0.7-kb *Eco*RI-*Pst*I fragment from the 3′ end of the human β-globin gene. The intact locus should give a band of 11.0 kilobases, indicated by arrow. W1–W6 are G418-resistant MEL clones transfected with wild-type μLCR^{A}γψβδβ-pWE15 cosmid. D1–D3 are clones transfected with the μLCR^{A}γψβδβ-del(*pyr* sequence)-pWE15 cosmid. MEL, untransfected MEL; HUM, human genomic DNA; C, μLCR^{A}γψβδβ-pWE15 cosmid DNA.

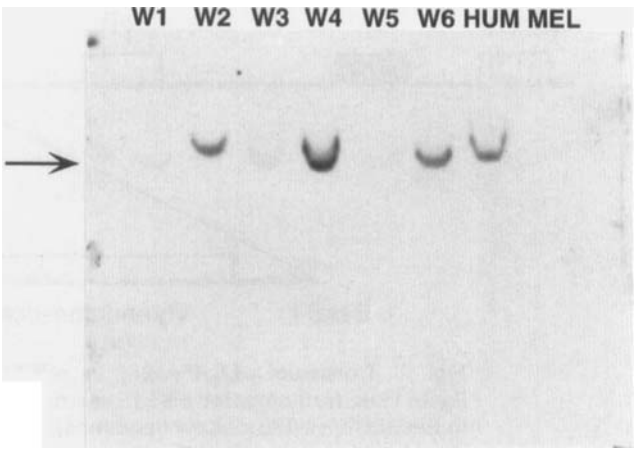

Fig. 3. Southern blot analysis of transfected clones using phage particle-mediated transfer of cosmid DNA. Ten micrograms of *Xba*I-digested genomic DNA were run on a 0.7% agarose gel, blotted onto nylon membrane, and probed with a 0.7-kb *Eco*RI-*Pst*I fragment from the 3′ end of the human β-globin gene. The intact locus should give a band of 11.0 kilobases, indicated by arrow. W1–W6 are G418-resistant MEL clones transfected with wild-type μLCR^{A}γψβδβ-pWE15 cosmid. HUM, human genomic DNA; MEL, untransfected MEL cells.

for the MEL cell ine by using Lipofectin reagent (Gibco BRL), a liposome formulation of two cationic lipids, to enhance the uptake of phage particles [15]. In two sets of transfections performed, there were approximately sevenfold and tenfold increases in the number of G418-resistant MEL clones obtained with this protocol, as compared to the original method that used cosmid DNA and calcium phosphate coprecipitation. Southern blot analysis of the cells transfected with naked cosmid DNA revealed that several G418-resistant clones contained rearrangements or deletions of the cosmid, using the 3′ end of the human β-globin gene as a probe (Fig. 2). Analysis of clones in which the cosmid was introduced by phage transfer showed no evidence of loss or rearrangement of the human β-globin gene (Fig. 3) or γ-globin gene (data not shown). Thus, the phage particle transfer method allows efficient and stable transformation of MEL cells.

Primer extension analysis was performed to analyze gene expression in the clones obtained by phage-mediated DNA transfer. The expression of the human globin genes paralleled that of the endogenous mouse globin genes with DMSO induction (data not shown). There was continued expression of the human γ-globin gene in the wild-type cosmid in MEL cells (Fig. 4). This result is in contrast to the situation in adult murine hematopoietic cells in transgenic mice with this construct, in which human γ-globin gene expression is largely silenced [4]. The majority of wild-type clones examined, however, expressed less γ globin as compared with human β globin. MEL clones transfected with the *pyr* sequence-deleted cosmid were also analyzed. There were equal amounts of human β and γ globin transcripts in most of these clones (Fig. 5). No lines examined showed elevated human β over γ expression as was found in most of the wild-type clones. However, the variability of results from clone to clone does not permit a conclusion regarding a difference in relative ^{A}γ and β globin expression in MEL cells containing the wild-type and deleted cosmids.

DISCUSSION

The expression of a cosmid construct that contains critical restriction fragments derived from the LCR linked to the human ^{A}γ, δ, and β globin genes in their natural context has been examined in mouse erythroleukemia cells. The globin sequences obtained from this cosmid have been shown to be expressed in a developmental stage-specific manner in transgenic mice [4]. We sought to determine if this regulation was retained in this erythroid cell line. Previous studies transfecting γ genes without the LCR showed variable expression in MEL cells [16–18]. Addition of hypersensitive site 2 of the LCR to a cosmid construct containing both fetal and adult genes failed to show suppression of γ gene expression in these cells [5]. Our results confirm these findings, using all four hypersensitive domains of the LCR, and indicate that a full complement of LCR-hypersensitive sites does not silence the human γ gene in MEL cells.

There are several possible explanations for the lack of suppression of human γ gene expression from our cosmid

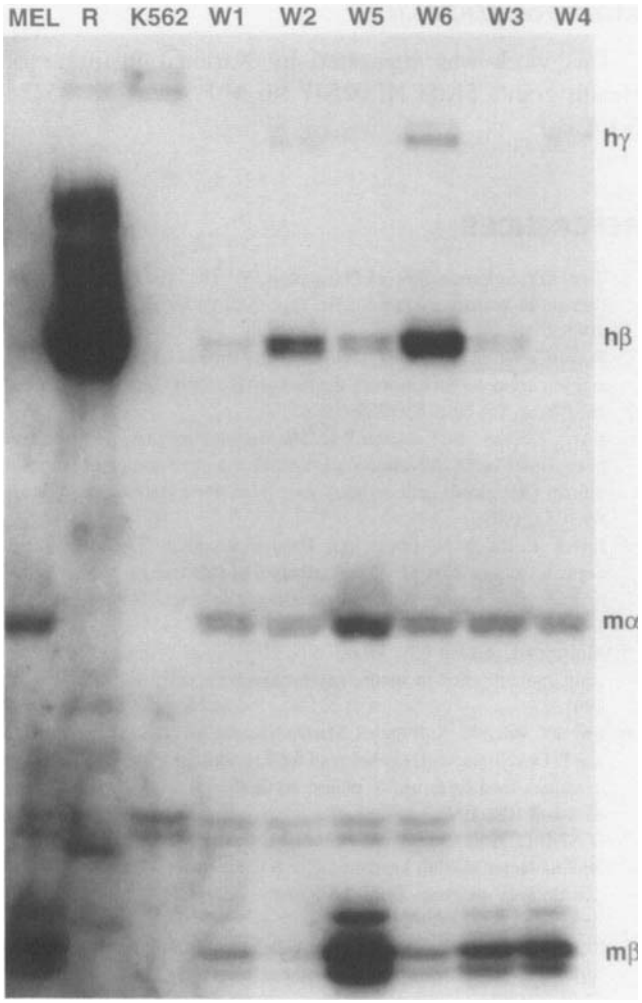

Fig. 4. Primer extension analysis of RNA from DMSO-induced MEL clones transfected with wild-type μLCR^{A}γψβδβ-pWE15 cosmid. The products were analyzed by electrophoresis on a 6% polyacrylamide gel under denaturing conditions. MEL, untransfected MEL cells; R, human reticulocyte; K562, hemin-induced K562 cells; W1–W6, MEL clones transfected with the cosmid. cDNA products resulting from reversed transcribed mRNA are indicated at right (mα, mouse α globin; mβ, mouse β globin; hβ, human β globin; hγ, human γ globin). Note that the ^{A}γ transcript is smaller in the transfected cells because the gene was modified in the original cosmid construct [4].

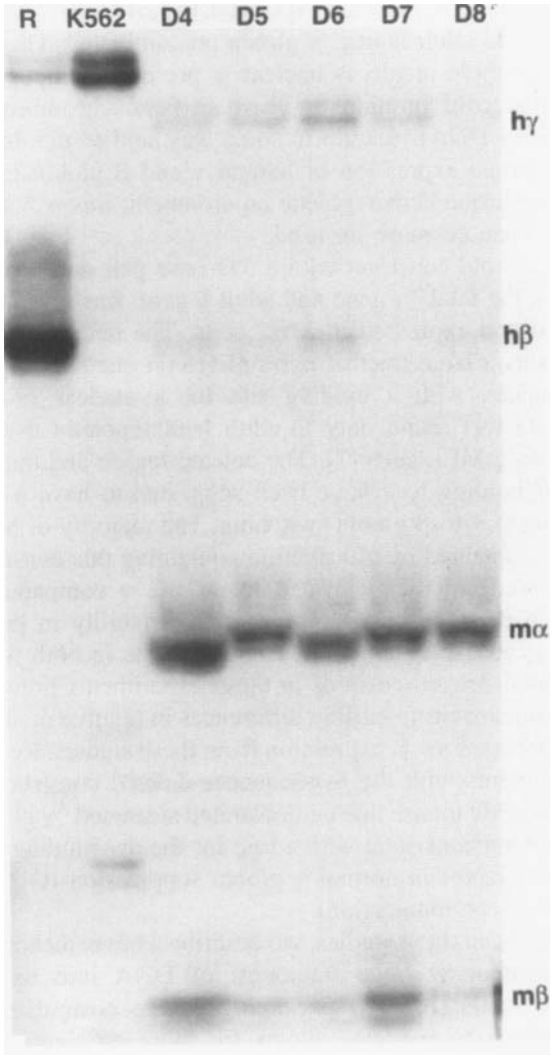

Fig. 5. Primer extension analysis of RNA from DMSO induced MEL clones transfected with the mutant μLCR^{A}γψβδβ-del(*pyr* sequence)-pWE15 cosmid. The products were analyzed by electrophoresis on a 6% polyacrylamide gel under denaturing conditions. R, human reticulocyte; K562, hemin-induced K562 cells; D4–D8, MEL clones transfected with the cosmid. The cDNA products resulting from reversed transcribed mRNA are indicated at right (mα, mouse α globin; mβ, mouse β globin; hβ-, human β globin; hγ, human γ globin).

construct in MEL cells. The construct contains only a portion of the locus control region, and does not include potential regulatory sequences distal to the β globin gene. However, this explanation is unlikely, since our construct has been shown to exhibit appropriate switching of fetal to adult globin in transgenic mice [4]. Alternatively, the use of MEL cells as representative of adult human erythropoiesis may not be justified. MEL cells can be induced to express embryonic mouse globin ϵ^{y} with sodium butyrate [19], whereas this gene is completely silenced in adult mice. Thus, there may be developmental stage-specific modification of DNA structure occurring in mouse erythroid development, but not in MEL cells, that is critical for the silencing of human γ gene expression. The demonstration that a human γ-globin gene was expressed at all stages of development in human fetal × MEL cell hybrids, whereas chromosomally introduced globin sequences showed appropriate switching, supports this hypothesis [6]. Transfer of a yeast artificial chromosome (YAC) containing 230 kb of the globin locus via fusion of YAC-containing L cells with MEL cells has also been reported [20]. Early after hybrid formation,

human ε and γ genes are expressed, but after 10–20 weeks in culture, adult human β globin predominated. The reason for these results is unclear at present. It is possible that the configuration of yeast artificial chromosomes modifies DNA structure in some way, and results in the appropriate expression of human γ and β globin genes in the murine hematopoietic environment; this is not the case when cosmids are used.

A cosmid construct with a 511-base pair deletion between the fetal $^{A}\gamma$ gene and adult δ gene was also introduced and expressed in MEL cells. The deleted region contains a large tract of pyrimidines on one strand (*pyr* sequence), with a binding site for a nuclear protein (*pyr* factor) found only in adult hematopoietic tissues, including MEL cells [7]. The deleted region and the *pyr* factor binding to it have been suggested to have a role in human γ-to-β globin switching. The majority of MEL clones obtained by transfection containing this construct expressed equal or elevated levels of $^{A}\gamma$ compared to human β transcripts. However, the variability in levels of γ gene expression from clone to clone in both wild-type and deleted cosmids in these experiments prevents any conclusions regarding differences in relative or absolute human γ vs. β expression from these studies. Recent experiments with the *pyr*-sequence-deleted construct in a transgenic mouse line demonstrated sustained $^{A}\gamma$ globin expression consistent with a role for the *pyr* binding site and *pyr* factor in normal γ globin suppression (O'Neil, personal communication).

Finally, in these studies, we describe a novel technique for introducing large fragments of DNA into tissue-culture cells. In vivo packaging of the cosmids into bacteriophage particles allows for more efficient gene transfer when compared with $CaPO_4$ precipitation of naked DNA into the MEL cell line. This method eliminates the need for production of large quantities of purified cosmid. No gross deletions or rearrangements of the β or γ genes are observed in the MEL cell transformants using phage packaging. This technique is currently being applied to introduce these cosmids into undifferentiated embryonic stem cells with the goal of following the expression of human globins during mouse hematopoietic development.

CONCLUSIONS

We describe a novel technique, employing phage particles and lipofection, to introduce large fragments of DNA into cells. Mouse erythroleukemia (MEL) cells can express the human γ globin gene when transferred by a cosmid construct that also contains components of the LCR and the human δ and β globin genes. The clonal variability in expression of these genes does not allow analysis of mutations in this construct.

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